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Transcriptome Analysis of the *Lactococcus lactis* ArgR and AhrC Regulons[∇]

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In previous studies, we have shown that direct protein-protein interaction between the two regulators ArgR and AhrC in *Lactococcus lactis* is required for arginine-dependent repression of the biosynthetic *argC* promoter and the activation of the catabolic *arcA* promoter. Here, we establish the global ArgR and AhrC regulons by transcriptome analyses and show that both regulators are dedicated to the control of arginine metabolism in *L. lactis*.

In many bacteria, arginine metabolism is controlled by transcriptional regulators of the ArgR family, including the well-characterized ArgR of *Escherichia coli* (13), AhrC of *Bacillus subtilis* (5), and ArgR of *Bacillus stearothermophilus* (7). Regulation is mediated through the interaction of the regulators with operators designated ARG boxes, which consist of 18-bp palindromic structures overlapping the core promoters of target genes (4, 5, 17). Low-G+C-content gram-positive bacteria often harbor more than one ArgR-type homologue (see reference 2 for an overview), and separate studies recently demonstrated that two arginine regulators are necessary for the regulation of arginine metabolism in the lactic acid bacteria *Lactococcus lactis* and *Lactobacillus plantarum* (11, 15). The deletion or mutation of any single arginine regulator in these organisms results in the complete disruption of arginine-mediated regulation. *L. lactis* AhrC is known to facilitate the binding of ArgR to ARG boxes in the biosynthetic *argC* promoter and to sequester and prevent ArgR from binding to ARC boxes (ARG box half sites) in the catabolic *arcA* promoter (12). In this way, arginine leads to the repression of arginine biosynthesis and the activation of arginine catabolism.

Here, we establish the complete regulons of ArgR and AhrC in *L. lactis* and show that these regulators are dedicated to the control of arginine metabolism.

ArgR and AhrC are dedicated regulators of arginine metabolism. The global regulatory effects of ArgR and AhrC of *L. lactis* were studied by DNA microarray-based transcriptome analyses as described previously, by using amplicon-based *L. lactis* IL-1403 slides (6, 10). IL-1403 amplicon sequences and the microarray data used are available online at http://molgen.biol.rug.nl/publication/arg_data/. RNA was isolated from cells grown to mid-exponential phase in chemically defined medium (CDM)

containing 10 mM arginine (11). The transcription profile of *L. lactis* MG1363 was compared to those of its two single regulator mutants MGΔ*argR* and MGΔ*ahrC* and the double regulator mutant MGΔ*argRahrC* (11). Significant derepression of all the genes in the *argCJDBF*, *gltS-argE*, and *argGH* operons of the arginine biosynthesis pathway in all three mutants was observed (Fig. 1). These results extend the known ArgR/AhrC regulons considerably and also establish the operon structures of the regulated genes, in agreement with the predicted promoter and putative terminator structures (Fig. 1). Whereas the levels of derepression of all biosynthesis genes in MGΔ*argR*, as well as those in MGΔ*argRahrC*, varied between 5- and 15-fold, the levels of derepression of these genes in MGΔ*ahrC* never exceeded 6-fold (Fig. 1). The lower mutant-versus-wild-type ratios obtained for MGΔ*ahrC* than for MGΔ*argR* and MGΔ*argRahrC* can be explained by the previous observation that ArgR, independently of AhrC, can weakly bind to the *argC* promoter in the presence of arginine (12). Still, both arginine regulators are essential for the complete repression of arginine biosynthesis, as the loss of either of the two regulators leads to an increase in the expression of all involved genes.

Regulation of the arginine catabolic gene cluster. The transcriptome analyses demonstrated a complex pattern of regulation of the *arc* gene cluster encoding the catabolic arginine deiminase (ADI) pathway (Fig. 2). The deletion of *argR* had no significant effect on *arc* expression, while the deletion of *ahrC* resulted in general downregulation, and the expression of *arc* genes in MGΔ*argRahrC* was increased relative to that in the wild-type strain (Fig. 2A). The *arcB* gene did not show significant changes in expression in any of the regulator mutants (Fig. 2A), which seems surprising considering that the changes in the expression of the other *arc* genes were highly significant. *L. lactis arc* is recognized as one of the largest and most complex of bacterial ADI pathway operons (19). The ADI operon *arcDABC* of *Pseudomonas aeruginosa* is subject to post-transcriptional regulation via mRNA processing and transcription termination, resulting in differential gene expression according to the requirement of the gene products in the cell (8, 9). A similar process may be in effect in *L. lactis*, although the

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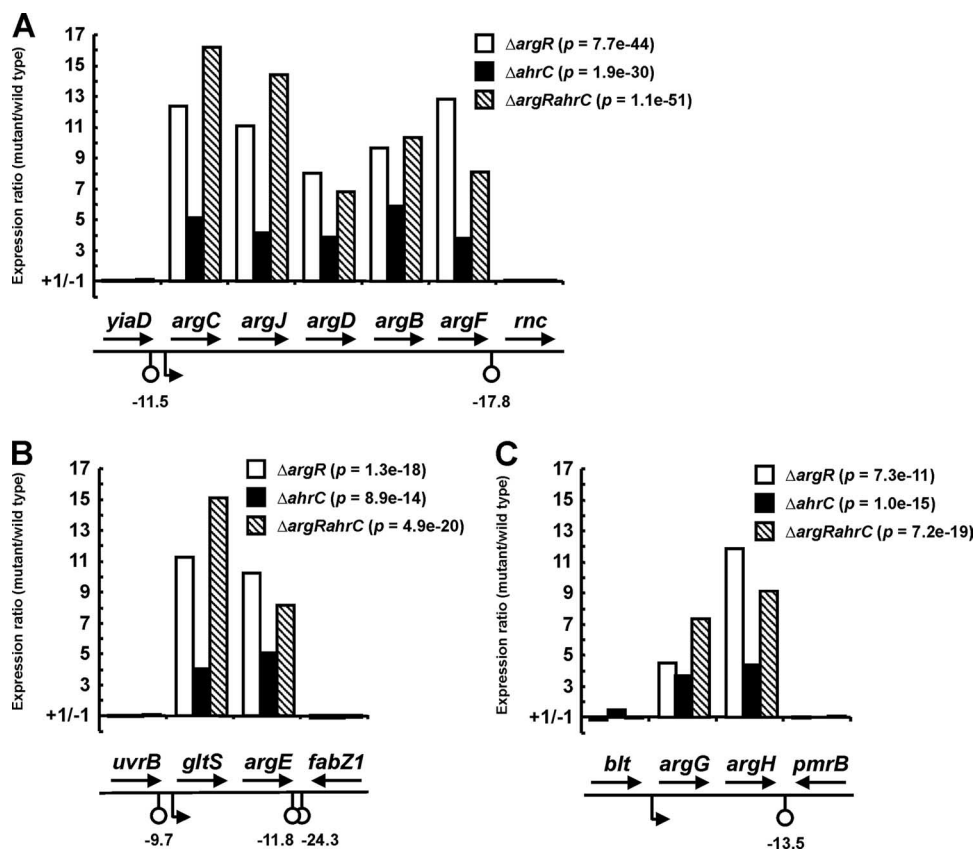


FIG. 1. Derepression of the arginine biosynthesis operons of *L. lactis*, as measured by DNA microarray analyses. Cells were grown to mid-exponential phase in CDM with 10 mM arginine (11). The *argCJDDBF* (A), *gltS-argE* (B), and *argGH* (C) operons and their neighboring genes, predicted promoters, and putative stem-loop (terminator) structures (calculated as ΔG° in kilocalories per mole) are drawn schematically, not to scale. The bars in the graphs show the ratios of expression in *L. lactis* strains MG $\Delta argR$, MG $\Delta ahrC$, and MG $\Delta argRahrC$ relative to that in *L. lactis* MG1363. The change in expression (n -fold) is shown on the y axis, with positive and negative values indicating up- and downregulation, respectively; if the expression of a gene in the mutant was equal to that in the wild-type strain, this resulted in a value near the x axis (+1/-1). The combined P values for the respective operons, thus excluding the values for surrounding genes, are given in parentheses next to the symbols.

role therein, if any, of the putative stem-loop structures downstream of *arcB* (Fig. 2A) remains to be investigated.

RT-PCR analysis of the *arc* gene cluster. To verify the *arc* operon structure as determined by transcriptome analyses, reverse transcription (RT)-PCR analyses of total RNA from cells grown in CDM with 10 mM arginine were carried out using primer pairs spanning all intergenic and neighboring regions. Only a little RT-PCR product corresponding to the *arcT-arcD2* and *arcD2-yvaD* regions was formed, while no product was obtained by probing between *argS* and *arcA* and between *yvaD* and *dinP*, defining the arginine catabolic operon to comprise *arcABD1C1C2TD2-yvaD* (Fig. 2B). The lower level of transcription of promoter-distal genes than of genes further upstream in the operon is likely caused by the possible terminator ($\Delta G = -19.2$ kcal/mol) in the *arcT-arcD2* intergenic region, which would allow only partial transcription elongation (Fig. 2). The putative *yvaD* product does not show homology to any known protein, and its function was not investigated further.

ARG box operators in *L. lactis*. Overrepresented sequence motifs in the regions upstream of the ArgR and AhrC target genes were identified using the MotifSampler online tool (18). Considering the regulatory patterns and the resulting operon structures (Fig. 1 and 2), only the regions upstream of the first

genes of the operons were subjected to the motif search. By employing the Genome2D software tool (1), an 18-bp motif was found to be present in all three arginine biosynthetic promoters (Fig. 3). A weight matrix based on the lactococcal ArgR motif was applied to all the promoter regions of *L. lactis* by using Genome2D (1). The obtained motif sequences were ranked according to their levels of similarity to the consensus sequence. By using the matrix, 8 of the 10 targets with the highest levels of similarity to the consensus sequence were determined to be present in the promoters of the arginine biosynthetic genes of *L. lactis* MG1363 (data not shown). The identified motif has a high degree of similarity to the 5'-WnT GnATWWWWATnCAW-3' consensus ARG box motif of *E. coli* (uppercase letters represent highly conserved residues) (13) (Fig. 3A). However, the typical 2- to 3-bp spacing between double ARG boxes in *E. coli* and other organisms was not observed in *L. lactis*, where the spacing (in multitudes of 10 to 11 bp) suggests the ARG boxes to be present on the same face of the helix but separated by one, two, or three helical turns (Fig. 3B). Note that no clear ARG box was obtained for the *arcA* promoter region, which is in agreement with results obtained previously by DNase I footprinting (12).

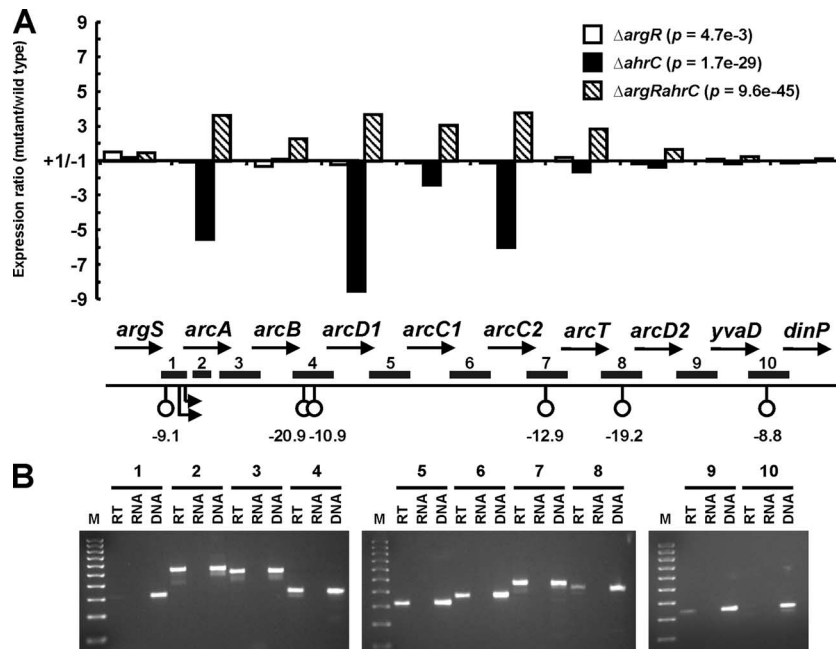


FIG. 2. (A) Deregulation of the *L. lactis* arginine catabolic *arcABD1C1C2TD2-yvaD* gene cluster (not drawn to scale). Refer to the legend to Fig. 1 for details. Numbered horizontal bars refer to regions examined by RT-PCR. (B) Results from RT-PCR investigation of the *arc* operon structure. Primer pairs were designed to amplify the intergenic regions indicated by numbered bars in panel A. Regions 1 and 2 correspond to RT-PCR probes of the *arcA* promoter region (negative control) and an intragenic *arcA* region (positive control), respectively. "DNA" indicates a positive PCR control with chromosomal DNA as the template. "RT" and "RNA" indicate PCRs performed with total RNA with and without reverse transcriptase treatment, respectively, the latter acting as a negative control. M, molecular size markers.

Indirect effect on the pyrimidine genes of arginine regulator mutants. The levels of expression of pyrimidine de novo biosynthesis pathway genes (*pyr*) in $MG\Delta argR$ and $MG\Delta argRahrC$ were between 1.3- and 1.7-fold higher than those in strain

MG1363, whereas no significant effects in $MG\Delta ahrC$ were observed (Fig. 4; data are shown only for the *pyrR-carA* operon). To assess the validity of these DNA microarray results, the activities of orotate phosphoribosyltransferase (PyrE, the gene for which was upregulated 1.7- and 1.5-fold in $MG\Delta argR$ and $MG\Delta argRahrC$, respectively) and orotidine-phosphate decarboxylase (PyrF, the gene for which was upregulated 1.5-fold in both $MG\Delta argR$ and $MG\Delta argRahrC$) were determined (Table 1). The activities of these two enzymes of the pyrimidine de novo pathway confirmed the results of the transcriptome analyses, showing that the impaired arginine regulation in the two mutants lacking ArgR did have a significant albeit weak effect on downstream pyrimidine metabolism (Table 1). The *pyr* gene expression profiles correlated well with the expression profiles of the arginine biosynthesis genes and

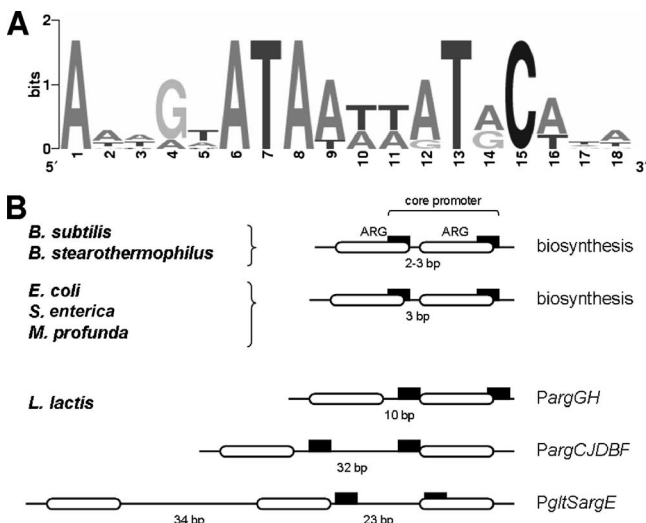


FIG. 3. *L. lactis* ARG operators. (A) Consensus logo of ARG box operators in the arginine biosynthetic promoters of *L. lactis*, created using the WebLogo tool (<http://weblogo.berkeley.edu/>) (3, 16). (B) Schematic comparison of ARG box organization patterns in different organisms (*S. enterica*, *Salmonella enterica* serovar Typhimurium; *M. profunda*, *Moritella profunda*). Core promoter -35 (left) and -10 (right) sequences are indicated as black boxes. White boxes represent 18-bp ARG boxes. The numbers below the horizontal lines (indicating chromosomal DNA) refer to the lengths of the spacer regions between ARG boxes.

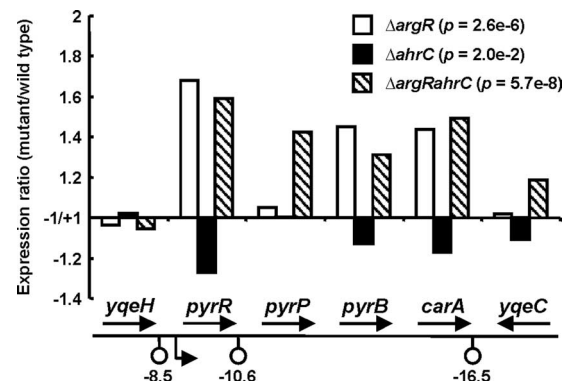


FIG. 4. Deregulation of pyrimidine de novo biosynthesis genes in the *pyrRBP-carA* operon of *L. lactis*. See the legend to Fig. 1 for details.

TABLE 1. Specific activities of orotate phosphoribosyltransferase (PyrE) and orotidine-phosphate decarboxylase (PyrF) in MG1363 and its isogenic regulator deletion derivatives^a

Strain	Specific enzymatic activity (U/mg of protein) (upregulation [<i>n</i> -fold]) ^b of:	
	PyrE	PyrF
MG1363	88 ± 17 (1)	197 ± 3 (1)
MGΔ <i>argR</i>	200 ± 37 (2.3)	256 ± 15 (1.3)
MGΔ <i>ahrC</i>	110 ± 31 (1.3)	146 ± 24 (−1.3)
MGΔ <i>argRahrC</i>	221 ± 76 (2.5)	251 ± 26 (1.3)

^a Cells were grown to mid-exponential phase in CDM with 10 mM arginine. Values shown are the averages and standard deviations determined from results obtained in three independent experiments.

^b Degree of upregulation in the indicated strain compared to the activity in the wild-type strain (MG1363).

not with those of the catabolic genes. Thus, in MGΔ*argR* and MGΔ*argRahrC*, the flux toward arginine was most likely increased due to the high-level expression of the arginine biosynthetic genes. As a consequence, carbamoylphosphate, which is required for the synthesis of both arginine and pyrimidine, was directed toward arginine, resulting in carbamoylphosphate and pyrimidine depletion. To counteract this effect, the pyrimidine biosynthetic genes, including both *carA* and *carB* (the latter is upregulated 1.9- and 1.5-fold in MGΔ*argR* and MGΔ*argRahrC*, respectively), were induced, possibly via the known transcriptional regulator PyrR (14).

Concluding remarks. The presence of two to four ArgR-type regulators in many low-G+C-content gram-positive bacteria led us to speculate as to possible global target genes of the two homologues in *L. lactis*. In support of the findings of our previous mechanistic studies (11, 12), we here show that both regulators are dedicated to the functional control of arginine metabolism. Additionally, the data extend the known ArgR/AhrC regulons of *L. lactis*, define the biosynthetic (*arg*) and catabolic (*arc*) operon structures, and show that even weak indirect effects can be significantly measured for the de novo pyrimidine genes in response to disrupted arginine regulation.

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